PATENT
4518-0101P

IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT:	Eckert, Helmut et al.	CONF:	8183
SERIAL NO.:	10/090,663	GROUP:	1642
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FOR:	MONOCLONAL ANTIBODIES AND THEIR USE		

DECLARATION SUBMITTED UNDER 37 C.F.R. § 1.132

Honorable Commissioner
Of Patents and Trademarks
Washington, D.C. 20231

July 13, 2004

Sir:

I, Dr. Günter Waxenecker of Igenson Krebs-Immuntheraple Forschungs- und Entwicklungs- AG, Vienna, Austria, do hereby declare the following:

I have attached a copy of my curriculum vitae to this Declaration.

I am Project Manager and conducted the experiments described below.

I am familiar with the above referenced patent application, as well as the development, usages and properties of anti-idiotypic antibodies.

I have read and understand the subject matter of the Office Action of January 16, 2004

The following comments are offered in support of the patentability of the instant invention.

Experimental evaluation of the screening method according to US 10/090663 for the selection of internal image anti-idiotypic antibodies to monoclonal antibodies BR55-2.

Summary

It has been shown by LeY ELISA that Ab1 binding to synthetic LeY is not inhibited by all products of murine hybridomas derived from immunization with Ab1 (=Ab2). Exclusively anti-idiotypic antibodies with internal image properties are able to inhibit Ab1 binding to the synthetic LeY antigen.

Abbreviations

Ab	antibody
Anti-id	antibody specific for the idiotype (binding site) of another antibody
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell scanning
IAC	immune affinity chromatography
IEX	ion-exchange chromatography
Ig	immunoglobulin
IGN301	drug product = drug substance (anti-idiotypic antibody MMA 383) precipitated on Alhydrogel
LeY	Lewis Y
Mab	monoclonal antibody
OD	optical density
PBS	phosphate-buffered saline
SEC	size exclusion chromatography

Introduction

In order to obtain anti-idiotypic antibodies against BR55-2, mice were immunized using F(ab')2-fragments of the IgG3 subtype of BR55-2 (ABL 364) coupled to KLH. This conjugate enhances the immunogenicity of the murine Ab1 in mice and the F(ab')2-fragments reduce the occurrence of antiisotype antibodies. Subsequent fusion of the spleens of these mice with the myeloma cell line SP2 led to hybridomas producing anti-BR55-2 antibodies. The screening strategy for anti-idiotypic mAb's is based on the following parameters:
Pre-selection of hybridoma supernatants by measurement of the binding to F(ab')2-fragments (ELISA) and by a cell-ELISA system with LeY positive breast cancer cell line SKBR5 used to detect the inhibition of the binding of BR55-2/IgG2a subtype to this cell line by hybridoma supernatants in the presence of

excess of irrelevant mouse-IgG. This test is expected to give indications for "internal image" anti-id mAb's.
In order to show, that products derived from hybridoma fusions after immunization with Ab1 do not exclusively yield "internal image" properties the 2nd assay has been performed with three subclones (3C12, 2F11 and 4F2) after first selection (binding to $F(ab')_2$-fragments of Ab1). Per definition antibodies without the internal image properties do not have the quality to inhibit Ab1 binding to the antigen. This test was performed in a quantitative manner based on the determined IgG concentration. Purified antibodies from subclones after first selection were tested again for indications of internal image properties of Ab2 by inhibition assay as mentioned above, but based on synthetic LeY ELISA instead of LeY antigen positive SKBR5 human breast cancer cells.

Methods

Fermentation and cell culture

Media: DMEM, ATCC, Cat. No. 30-2002
Nutridoma SP, Roche, Cat. No. 1011375
FCS, Gibco; Cat. No. 10500-064
Hybridoma Fusion and Cloning Supplement, Roche, Cat. No. 1363735

Hybridoma cells 3C12, 4F2 and 2F11 (anti-Id BR55-2) secreting MMA 383 stored in liquid nitrogen were thawed, diluted with medium (DMEM+20%FCS+2%Nutridoma+2%HFCS) and centrifuged at 300 g for 10 minutes. The pellet was suspended in 10 ml medium, poured into a 25 cm^2 Roux cell culture flask, expanded to a volume of 40 ml and incubated at 37°C with 5% CO_2 in a 75cm^2 roux flask.

Down stream processing

Cell culture debris was separated by centrifugation (Beckman Coulter, 3500 rpm for 30min at 4°C). Supernatant was sterile filtered by Stericup (Millipore, 0,22µm) and stored at + 4°C until purification. After 1:3 or 1:10 dilution with PBS+0.2 M NaCl the dilution was sterile filtered again.

EasyTiter Mouse IgG Assay Kit

This assay is based on the use of purified antibodies coated onto polystyrene particles. For the measurement of Mouse IgG the kit contains Goat anti-Mouse IgG-coated particles. When these particles react with IgG in a test sample, they aggregate and no longer absorb light to the same degree. This absorbance can be measured using an ELISA reader. The yellow colour of the beads allows OD measurements to be performed at various wavelengths. Pierce recommends that measurements be taken at 405 nm or 340nm. Because this is an aggregation

assay, the standard curve yields high OD values at low IgG concentration and low OD values at high IgG concentrations.
The assay has been performed according to the manufacturer's protocol (Pierce Kit: #23300). MMA383 #98802 has been used as standard.
Standard and samples were generated in duplicates. Samples were applied in appropriate dilutions (1:10 in 6 steps to 1:107) undiluted and 1 to 10 further dilution steps
Two dilutions per Buffer were generated. In order to take account of the matrix effect standard curves were generated with the manufacturer's dilution buffer, the neutralized NH_3 and glycine elution buffer. The standard curve was generated by addition of 500 to 0 ng per ml buffer (seven equidistant dilution steps).

Protein A chromatography

The Protein A column (Amersham Pharmacia) was washed with binding buffer (PBS+0.2 M NaCl). After equilibration conditioned medium containing MMA 383 was loaded onto the column at a flow rate of 1ml/min. The gel was washed with binding-buffer below 30 mAU. After elution (2 CV) of the breakthrough fraction, the bound MMA 383 was desorbed with elution buffer (100mM glycine+0.2 M NaCl) and neutralized immediately after desorption with 1 M $NaHCO_3$ at pH 7.5. Protein elution was generally followed by absorbance at 280 and 214 nm. Vivaspin columns (Cat. No. VS02V1; Vivascience) with a MWCO of 10kD were used for concentration and buffer exchange from elution buffer to PBS (three times of the initial volume of buffer exchange). The concentration was determined by SEC.

SEC

For the determination of the IgG concentration of the samples, a standard curve using Pentaglobin has to be performed according to SOP (No.: 082PC021e.01).

Table 1: Method of the SEC used for the quantification of IGN311 for spiking and recovery

Column:	ZORBAX GF 250 (PN: 884973.901)
Running buffer:	200 mMol $NaPO_4$-Buffer pH=7 , 10% Acetonitrile
flowrate:	1,000 ml/min
wavelength:	214 nm und 280nm
bandwidth:	6 nm
Injection volume:	variabel von 100µl
Running time:	19.00 min

LeY ELISA

1 µg IgG (concentration determined by SEC) per ml with 10 µg irrelevant mouse IgG (Sigma Cat. No.I-5381) was incubated for 30 min at 37°C. 50 ng BR55-2/mIgG2a (880-365) were added to the mixture and incubated for 30 min at 37°C. 100 µl of this mixture were applied to LeY-PAA coated microtiter plates (5 µg LeY-PAA per ml coating buffer; 100 µl for 1 h at 37°C) and incubated for 1 hour. After a washing step binding of BR55-2/mIgG2a to LeY-PAA coated ELISA plates were detected by anti mouse IgG2a-HRP conjugate (HRP-Goat-anti Mouse IgG2a, Southern Biotechnology, Cat. No. 1080-05). After a washing step the substrate OPD (Sigma, Cat. No. P-8287) was added. After about 10 min incubation staining procedure was stopped by adding 50µl H_2SO_4 (30%).

BR55-2/mIgG2a in the presence of 10 µg irrelevant mouse IgG was used as positive control and was used to generate a standard curve. Binding of BR55-2/mIgG2a to LeY coated microtiter plates was tested in the presence of various concentrations of irrelevant mouse IgG (100 to 5 µg per ml). Rabbit anti mouse IgG was used as positive control in concentrations from 1 to 10 µg per ml in order to test the inhibition of LeY binding.

Results

160 ml supernatant of murine hybridomas 2F11 and 4F2 and 240ml of supernatant of clone 3C12 were produced and the concentration of mouse IgG were determined by Easytiter. After purification by Protein A chromatography, concentration and buffer exchange 668, 694 and 608 µg were yielded from clone 3C12, 2F11 and 4F2 respectively. These samples were tested at 1 µg per ml in a quantitative manner in the presence of 10 µg irrelevant mouse IgG for the capacity to inhibit LeY binding of BR55-2/murine IgG2a to LeY. Clinical grade drug substance #96802 derived from subclone 3H4 and preclinical grade drug substance VC0103-20 (secreted from clone E4) was used as positive controls. E4 has been selected after subcloning 2F11. 3H4 is a serum-free adapted subclone of E4 and has been used for the production of clinical grade material.

Table 2: Standard curve of BR55-2/IgG2a (880-365)

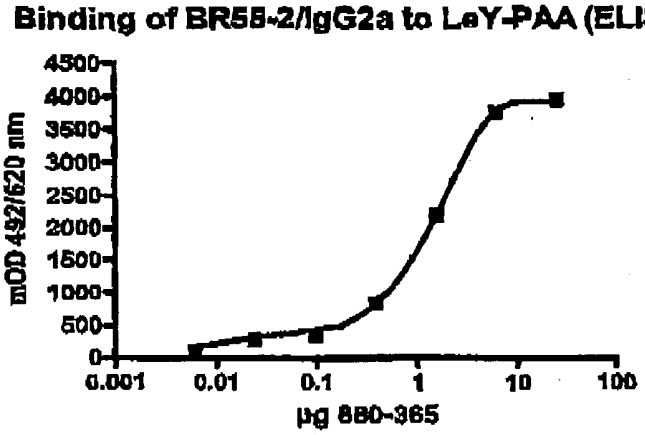

Table 3: Binding of LeY specific mAb 880-365 to synthetic LeY (ELISA) is fully inhibited by products from clone E4 and 3H4 and partially by purified antibodies of subclones 2F11 and 4F2 after first selection. No inhibition is measured with subclone 3C12 and isotype control MOPC21.

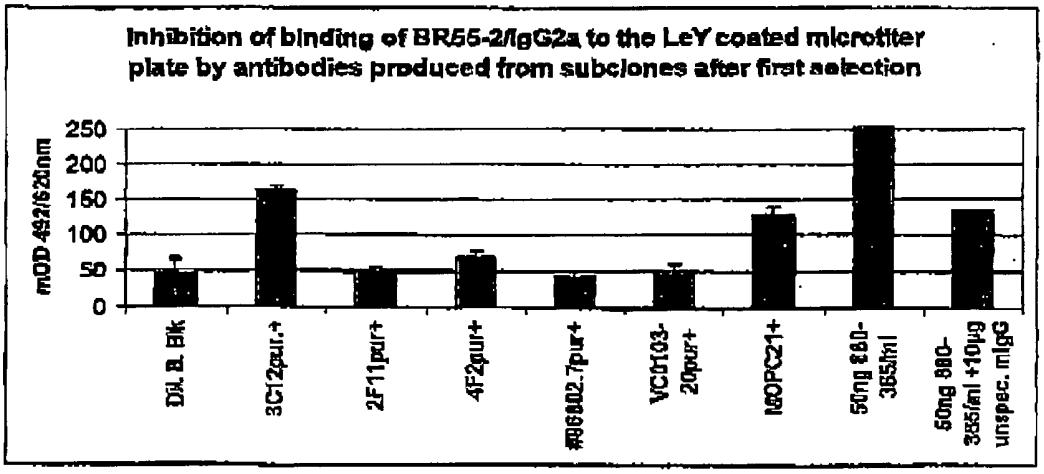

Table 4: % Inhibition of binding of BR55-2 to LeY coated ELISA plate

	% Inhibition
Dil. B, Blk	100
3C12	-28
2F11	86
4F2	75
#96802.7	105
VC0103-20	101
50ng 880-365/ml +10µg unspec. mIgG	0

Antibodies from clone 3C12 have no capacity to inhibit LeY binding of the LeY specific antibody 880-365 (-28%) other clone products have this property (4F2 and 2F11). 2F11 has been subcloned and after further screenings clone E4, and finally clone 3H4 were selected for further development. Products of both clones (#96802 and VC0103-20) fully inhibited LeY binding of 880-365 and consequently their internal image property has been confirmed.

Conclusions

Not all of the antibodies obtained by the immunization procedure have the capacity to sufficiently inhibit Lewis Y binding of the Lewis Y specific antibody 880-365 (BR55-2/mIgG2a). The screening method disclosed in the instant application allows the skilled artisan to isolate internal-image anti-idiotypic antibodies to monoclonal antibodies BR55-2 from other antibodies produced, and to obtain a population of antibodies each of which has an inhibition capacity of at least 95% in terms of inhibition of binding BR55-2 murine IgG2a to Lewis Y.

Note: The results obtained from the LeY ELISA system are directly comparable to results of a cell based assay for Lewis Y positive human breast cancer.

The undersigned hereby declares that all statements made herein based upon knowledge are true, and that all statements made based upon information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DATED: 16.7.04

Dr. Günter Waxenecker

Enclosures: As stated above